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Distorted copper homeostasis with decreased sensitivity to cisplatin upon chaperone *Atox1* deletion in *Drosophila*

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Abstract Copper is an integral part of a number of proteins and thus an essential trace metal. However, free copper ions can be highly toxic and every organism has to carefully control its bioavailability. Eukaryotes contain three copper chaperones; Atx1p/ Atox1 which delivers copper to ATP7 transporters located in the trans-Golgi network, Cox17 which provides copper to the mitochondrial cytochrome c oxidase, and CCS which is a copper chaperone for superoxide dismutase 1. Here we describe the knockout phenotype of the *Drosophila* homolog of mammalian *Atox1* (*ATX1* in yeast). *Atox1*^{−/−} flies develop normally, though at reduced numbers, and the eclosing flies are fertile. However, the mutants are unable to develop on low-copper food. Furthermore, the intestinal copper importer Ctr1B, which is regulated by copper demand, fails to be induced upon copper starvation in *Atox1*^{−/−} larvae. At the same time, intestinal metallothionein is upregulated. This phenotype, which resembles the one of the *ATP7* mutant, is best explained by intestinal copper accumulation, combined with insufficient delivery to the rest of the body. In addition, compared to controls,

Drosophila Atox1 mutants are relatively insensitive to the anticancer drug cisplatin, a compound which is also imported via Ctr1 copper transporters and was recently found to bind mammalian Atox1.

Keywords *Drosophila* · Atox1 · Copper · Ctr1B · Cisplatin

Introduction

All organisms, including eukaryotes from yeast to humans, use elaborate systems to regulate copper homeostasis (O'Halloran and Culotta 2000; Puig and Thiele 2002; Mercer and Llanos 2003; Balamurugan and Schaffner 2006; Kim et al. 2008). Copper is a trace metal that is both indispensable for normal cell function and potentially toxic. Due to its capacity to undergo redox cycling between Cu(I) and Cu(II), copper serves as a cofactor of redox enzymes, such as Cu/Zn-superoxide dismutase (Cu/Zn SOD), tyrosinase, lysyl oxidase and cytochrome c oxidase (COX), whose functions are compromised by copper deficiency. Several diseases are characterized by an aberrant copper metabolism. In Menkes disease, mutations in the copper transporter *ATP7A* gene result in an insufficient peripheral supply of copper (Mercer 2001). Other mutations in the *ATP7A* gene cause occipital horn syndrome (OHS) or distal motor neuropathy (DMN) (Tumer et al. 1999; Kennerson et al. 2010). A defective copper homeostasis has also

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been implicated in Alzheimer's disease (AD), where patients were reported to have a reduced copper content in the brain (Maurer et al. 2000; Cottrell et al. 2001; Bayer et al. 2003).

In all eukaryotes, copper is imported as Cu(I) by high-affinity copper transporters of the Ctr family (Petris 2004). Ctr proteins form homotrimeric complexes in the membrane and import copper in an ATP independent manner (Lee et al. 2002; De Feo et al. 2009). In the rapidly growing *Drosophila* larvae, copper uptake from the food is mainly executed by the copper transporter Ctr1B (Zhou et al. 2003; Selvaraj et al. 2005; Balamurugan et al. 2007). *Drosophila* has two additional Ctr's; Ctr1A is ubiquitously expressed at all stages of development at a relatively low level (Turski and Thiele 2007) and Ctr1C, which is strongly expressed in the male gonads. Ctr1C, in cooperation with Ctr1B, ensures adequate copper supply for germ cell formation and fertility (Steiger et al. 2010).

There are three types of copper chaperones that accept copper from Ctr proteins and transfer it to their specific target proteins (Markossian and Kurganov 2003): CCS, which delivers copper to SOD1 (Cu/Zn superoxide dismutase) (Schmidt et al. 2000; Wong et al. 2000; Culotta et al. 2006); Cox17 which, assisted by Sco1 and Cox11, transfers copper to the mitochondrial cytochrome c oxidase (Srinivasan et al. 1998; Horng et al. 2004); and Atox1/Atx1p. The ortholog of Atox1 in yeast, Atx1p, brings copper to Ccc2p, a copper transporting ATPase (Arnesano et al. 2001). Mammalian Atox1 delivers copper to the human Ccc2p homologs, ATP7A and ATP7B, which localize to the trans-Golgi network (Walker et al. 2002; Hamza et al. 2003). The *Drosophila* ortholog of yeast Ccc2p and mammalian ATP7A and ATP7B, termed DmATP7, has been characterized by Camakaris, Burke and colleagues (Norgate et al. 2006; Burke et al. 2008). It delivers copper to cuproenzymes and also serves to export excess cellular copper; accordingly, *DmATP7* mutants overaccumulate copper in the intestine but suffer from copper deficiency in other parts of the body.

Atox1 is conserved among eukaryotes, from mammals to insects and fungi (Fig. 1a). An ortholog of Atox1, called CopZ, was even identified in bacteria (Cobine et al. 1999), where it delivers copper to the ATP7 homolog CopA. In mice, lack

of Atox1 leads to copper deficiency with a high mortality shortly after birth. Surviving animals display a severe phenotype that includes growth failure, skin laxity and hypopigmentation (Hamza et al. 2001). In the absence of Atox1, copper accumulates in cultured mouse fibroblasts due to impaired copper efflux (Hamza et al. 2003). Since the molecular function of *Drosophila* Atox1 has not been elucidated so far, we decided to generate an *Atox1* null mutant. Our results show that *Atox1* mutant *Drosophila* are sensitive to copper starvation but, at the same time, relatively less susceptible to cisplatin treatment, a drug that is imported via Ctr1 type transporters (Lin et al. 2002; Holzer et al. 2004). Most likely in the *Atox1* mutant, analogous to the *DmATP7* mutant, copper accumulates in intestinal cells due to inefficient efflux of copper to the rest of the body.

Materials and methods

Fly stock maintenance

One liter of fly food is composed of 55 g corn, 10 g wheat, 100 g yeast, 75 g glucose, 8 g agar and 15 ml anti-fungal agent nipagin (15% in ethanol). Flies were raised and experiments were performed at 25°C and 60% humidity.

Generation of the *Atox1* null allele

An *Atox1* null allele (*Atox1*¹⁷⁴) was generated by imprecise excision of a single P-element insert 30 bp upstream of the transcription start site in the EP line EY15780 (BL21158, Bloomington Stock Center). The EP line turned out to be a hypomorph. EP flies were crossed to a line containing a transposase (*y w; +; Δ2–3 Sb/TM2*, a gift from Konrad Basler). 200 excision events were analyzed by PCR followed by sequencing. Among them, a 450 base pair deletion (*Atox1*¹⁷⁴) beginning at the EP insertion site was identified, which removed the first two of the three exons of the *Atox1* gene, whereas neighboring transcription units were not affected. A precise excision of the P-element without any deletion of fly DNA was also generated, and served as the wild type *Atox1* control for genetic background in all experiments.



Fig. 1 Generation of the *Atox1* null mutant. **a** Sequence alignment of copper chaperones that have been shown or suggested to transfer copper ions to ATP7-type transporters. Flybase CG32446 is the *Drosophila melanogaster* ortholog of human Atox1. The amino acids that were deleted in the *Atox1* mutant allele are underlined. **b** Genomic position of the

Drosophila Atox1 gene. Grey bars represent the neighboring genes, without depicting their exon/intron structure (data from Flybase), the gap in the solid line indicates the deletion generated by imprecise “jump-out” of the P-element EY15780. The scale bar corresponds to a 1 kb genomic region

Survival assay

For survival experiments, fly food was supplemented with CuSO_4 , BCS (bathocuproinedisulfonic acid disodium salt, Sigma B1125) or cisplatin (cis-diammineplatinum(II) dichloride; Sigma P4394) to the indicated concentrations. BCS is a metal chelator with a strong preference for Cu(I). To measure the survival rate, 100 eggs were transferred to a vial containing standard food (NF) or to food with the indicated supplements. The survival rate was calculated as the percentage of flies that enclosed from the eggs. Each bar represents the average of at least three vials.

Genomic rescue construct and an *Atox1* overexpression fly line

To generate a genomic rescue construct, the genomic region of the *Atox1* gene including 300 bp upstream of the transcription start and 2 kb downstream of the transcription unit was cloned into a pAttB vector (Bischof et al. 2007). The *Atox1* coding sequence tagged with mCherry at the C-terminus was cloned into the transformation vector pUAST-AttB. Plasmids were introduced into the *Drosophila* genome at an AttP landing site (line ZH-51D) using the Phi C31 integration system (Bischof et al. 2007). For expression of the Atox1-mCherry protein, the UAS-Atox1-mCherry flies were crossed with flies containing the transactivator *Gal4* driven by the ubiquitously active *actin 5c* promoter.

Fluorescent microscopy

Guts were dissected from Atox1-mCherry adult flies and fixed by 4% formaldehyde for 20 min. Confocal images were recorded on a Zeiss LSM710 microscope. The images were recorded at 10× magnification and with 5 s exposure time.

To analyze the expression level of the *MtnB-EYFP* reporter gene (Egli et al. 2006), guts of 1–2 day old female flies were dissected and EYFP expression was recorded with a Zeiss Axioplan microscope and a Zeiss Axioplan MRm camera without fixation of the tissue. To distinguish yeast autofluorescence in the gut from specific fly EYFP signal, images of the FITC and TRITC filter were merged digitally using ImageJ software. Exposure times were kept the same for all recordings.

S1 nuclease protection assay

To determine mRNA levels, 3rd instar larvae were collected on either standard food or food with the indicated supplements. Total RNA was extracted using the TRIzol reagent (Life Technologies) and nuclease S1 mapping of transcripts was performed as described previously (Weaver and Weissmann 1979). The gels were developed using FLA-7000 system and bands were quantified using ImageGauge software (Fuji Film). The transcripts of the endogenous *actin 5c* gene were measured and used for normalization of transcript levels.

Results

Atox1 mutant flies are sensitive to copper starvation

The *Drosophila* homolog of human Atox1 was identified previously (Southon et al. 2004). To analyze its molecular function in more detail we generated a null mutant allele (Fig. 1b). Starting from a *Drosophila* strain with an insert of a modified P-element in the *Atox1* promoter region (EP line EY15780), we attempted to obtain deletion mutants of the *Atox1* gene by imprecise “jump-out”. Indeed, among 200 excision events, one was found with a deletion of most of the transcription unit (*Atox1*¹⁷⁴) and others with larger or smaller deletions. There were also several precise excisions, one of which was subsequently used as a control for the genetic background. The level of *Atox1* transcripts in control larvae maintained on normal food (NF), on copper-containing food and on copper-depleted food was determined. The *Atox1* mRNA level on normal food was also measured in the EP line and in the *Atox1*¹⁷⁴ deletion mutant flies (Fig. 2a). The transcript levels of wild type *Atox1* were unaffected by copper-supplemented food (250 μ M CuSO₄) or by copper-depleted food (100 μ M copper chelator BCS). In the EY15780 line, which has a P-element insertion in the *Atox1* promoter region, *Atox1* transcript levels were dramatically reduced to one-tenth of controls. In *Atox1*¹⁷⁴ deletion mutant flies no *Atox1* transcripts above background level could be detected. *Atox1* mutants are viable and fertile under standard laboratory conditions, although their survival rate during development is reduced. The most obvious phenotype is that *Atox1* mutant larvae cannot tolerate copper starvation under conditions which pose no problem for *Atox1* wild type *Drosophila* (Fig. 2b). By contrast, copper supplemented food had no influence on the viability of *Atox1* mutant larvae (data not shown).

Expression patterns of tagged *Atox1*

To detect the endogenous expression pattern of Atox1 in *Drosophila*, a transgene with the genomic *Atox1* gene was generated in which a hemagglutinin (HA) tag was fused to the C-terminus of Atox1. This construct was integrated into the *Drosophila* genome via the phage C31 integration system at an AttP site

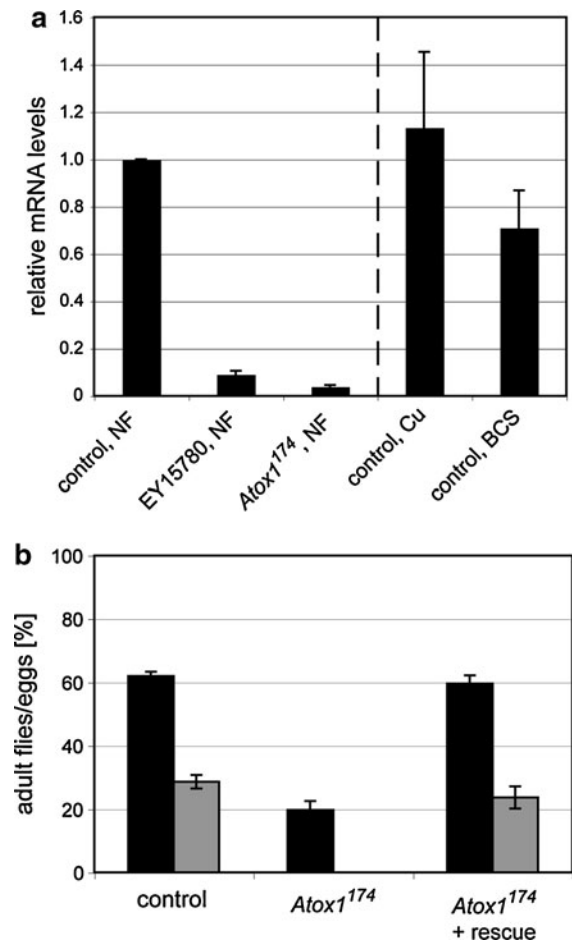


Fig. 2 *Atox1* mutant flies are sensitive to copper depletion. **a** *Atox1* mRNA levels were measured in control, EY15780 and *Atox1*¹⁷⁴ larvae. Controls were also subjected to copper load (250 μ M CuSO₄) and copper starvation (100 μ M chelator BCS). The low expression in EY15780 larvae with a P-element in the promoter of *Atox1* identifies this allele as a hypomorph, while transcripts in the deletion (*Atox1*¹⁷⁴) are at background level. **b** Under standard growth conditions (black bars) *Atox1* mutant larvae show reduced survival, while on food containing a copper chelator (250 μ M BCS; grey bars) they cannot develop at all. Resistance to BCS was restored by introducing a genomic *Atox1* rescue construct into the *Atox1* deletion mutant

located at genome position 51D and the expression pattern was visualized by immunostaining. In the adult gut, Atox1 is strongly expressed in enterocytes and another cell type which most likely represents intestinal stem cells, as inferred from their distribution and low degree of ploidy, typical for cells that are able to divide (data not shown). In another transgene an Atox1 fusion protein with the fluorescent protein mCherry was expressed under the control

of the ubiquitous transactivator *actin-Gal4*. In enterocytes of the gut, the Atox1-mCherry protein exhibited strong fluorescence with a punctate expression pattern (Fig. 3). By contrast, Atox1-mCherry levels were much lower when the flies were exposed to copper-supplemented food. In contrast to the situation in enterocytes, Atox1 levels were not affected by copper status in the small, low-ploidy, putative stem cells. This suggests that regulated expression is particularly important in enterocytes, since these are the cells that have to cope with fluctuating food copper levels.

Distorted copper homeostasis in larvae and adult flies lacking *Atox1*

The major copper importer in the larval gut, *Ctr1B*, is regulated at the transcriptional level: transcripts are induced at low copper conditions and repressed below basal level under conditions of excess copper (Zhou et al. 2003; Selvaraj et al. 2005). We used the S1-nuclease protection assay to monitor the transcriptional response of *Ctr1B* (Fig. 4a). While *Ctr1B* is up-regulated by copper starvation in *Atox1* wild type larvae, in *Atox1* mutants *Ctr1B* transcripts

remained low in larvae grown in BCS-supplemented food. Additionally, expression of a *MtnB-EYFP* reporter gene is stronger in *Atox1* mutant flies (Fig. 4b), supporting the idea that, in the absence of the specific chaperone, copper export via DmATP7 from intestinal cells to the rest of the body is impaired, with a concomitant copper accumulation in the gut (see “Discussion” section).

Atox1 mutant larvae are relatively insensitive to cisplatin

Cisplatin is a widely used anticancer drug, which after its uptake into cells is able to covalently bind to DNA, causing a DNA replication block and apoptosis of cells (Cepeda et al. 2007). It was previously found that copper importers of the Ctr type are major importers of cisplatin, even though the compound is structurally unrelated to copper ions. However, cisplatin uptake is apparently not executed via the same mechanism as copper import (Sinani et al. 2007). Since in mouse fibroblasts loss of *Atox1* leads to an increased resistance to cisplatin (Safaei et al. 2009), we also determined the sensitivity of *Atox1* mutant flies to cisplatin. Whereas wild type *Atox1*

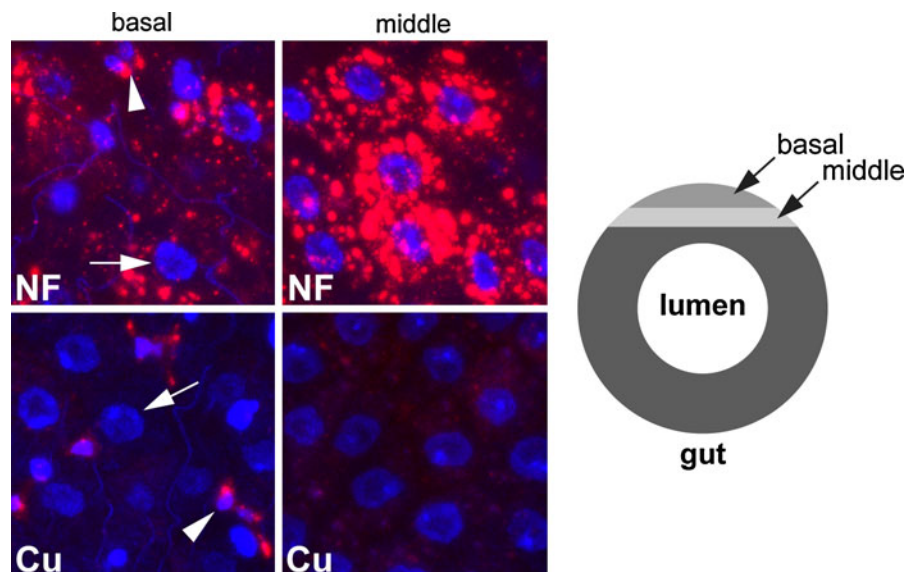


Fig. 3 Enterocyte Atox1 protein levels are reduced upon copper load. Under standard food conditions (NF), Atox1-mCherry shows a punctate staining in the intestinal cells of adult flies (upper row). When flies are kept on food containing 1 mM CuSO₄ for 3 days, staining intensity in enterocytes is substantially reduced (lower row). The graph on the right

illustrates the locations where the images were recorded. Close to the basal membrane of adult gut, there are both enterocytes and putative intestinal stem cells (with, depending on their level of ploidy, large (arrows) and small (arrowheads) DAPI-stained nuclei, respectively), whereas the middle region contains only enterocytes

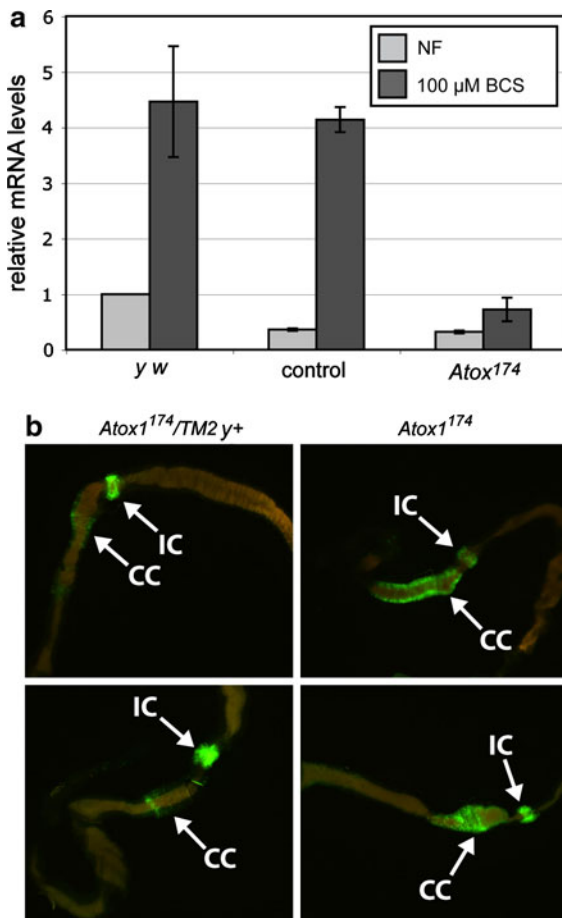


Fig. 4 Aberrant expression of copper importer *Ctr1B* in the *Atox1* mutant. **a** Quantification of *Ctr1B* transcripts by the S1 nuclease protection assay. In control animals (either a standard laboratory *y w* strain or animals resulting from a precise “jump-out” of the EY15780 P-element), *Ctr1B* expression is induced in the larval gut when they are grown in food with 100 μ M BCS. However, in the *Atox1* mutant background, the *Ctr1B* transcript levels were not significantly increased. **b** *Atox1* mutant flies show an upregulation of a metallothionein reporter gene (*MtnB-EYFP*) on NF most likely due to accumulation of intracellular copper, which is not seen in *Atox1* heterozygous flies (*Atox1⁷⁴/TM2 y+*). Autofluorescence of ingested yeast in the gut (orange) was visualized by overlaying the images of FITC and TRITC channels. CC copper cell region (intestinal cells which tend to hyperaccumulate copper); IC “iron cell” region. Iron cells also are specialized metal storing cells which preferentially accumulate iron and copper from the food

flies show a significantly reduced survival to adulthood on food containing 1 mM cisplatin, the viability of *Atox1* mutants was not affected by cisplatin at the same concentration (Fig. 5).

Discussion

Starting from a P-element insertion in the promoter region of the copper chaperone *Atox1*, we generated a null allele by imprecise excision. The fact that such flies are viable, albeit with a lower eclosion rate than control flies, and fertile, shows that the *Atox1* gene is not strictly required under standard laboratory conditions. However, our results clearly reveal a distorted copper homeostasis in *Atox1*-deficient *Drosophila*. While the mutants are not affected by copper load, they are highly sensitive to copper depletion, as they fail to develop on food supplemented with 250 μ M BCS copper chelator, a concentration which is readily tolerated by control flies (either *Atox1* wild type flies or *Atox1* mutants rescued with an *Atox1* transgene). *Atox1* is particularly abundant in the so-called “copper cells” in the midgut of both larvae and adult flies. These cells can accumulate excess amounts of copper upon copper load and readily lose their store upon copper depletion, apparently by transport via DmATP7, into the rest of the body (Balamurugan et al. 2007). While *Atox1* transcripts are hardly, if at all, changed by copper status of the flies, the *Atox1* protein level is reduced upon copper load: in transgenic flies expressing *Atox1*-mCherry protein, *Atox1* forms a strong punctate staining in enterocytes, while staining

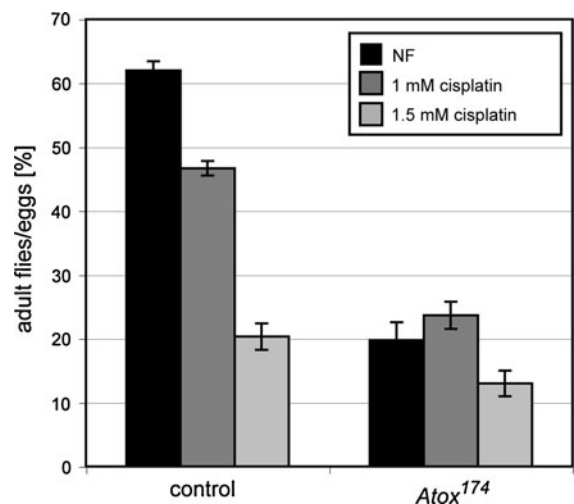


Fig. 5 *Atox1* mutant flies are less affected by cisplatin treatment than controls. Control flies show decreased viability with 1 or 1.5 mM cisplatin in the food. Although *Atox1* mutant flies generally develop at reduced numbers (see also Fig. 2b) they are less affected by these concentrations of cisplatin

is dull upon copper load. This indicates a posttranscriptional regulation, possibly at the level of translation efficiency and/or protein stability. To probe the possible interplay of Atox1 with the copper importer Ctr1B, or with the metalloregulatory transcription factor MTF-1, we also analyzed *Ctr1B* and *Atox1* double mutants, and *MTF-1* and *Atox1* double mutants. However, at least on normal food we did not observe any change in phenotype with either of these double mutants flies (data not shown).

The fact that expression of the major intestinal copper importer, *Ctr1B*, is not induced by copper depletion in the *Atox1* null mutant suggests that the intestinal cells have accumulated enough copper to exert a negative feedback on *Ctr1B* transcription. Consistent with a copper saturation of intestinal cells there is an elevated metallothionein (*MtB*) expression. Consequently, the lack of Atox1, which is the chaperone that normally escorts copper to the DmATP7 for transport to other parts of the body (Camakaris et al. 1999; Prohaska and Gybina 2004), would result in peripheral copper depletion. This scenario also explains the sensitivity of *Atox1* mutant flies to copper starvation. It is noteworthy that the gene for *DmATP7* is induced in the *Drosophila* midgut by copper in an MTF-1-dependent manner (Burke et al. 2008). Therefore, even an elevated level of this copper exporter, in the absence of its specific metal chaperone, obviously does not suffice as a measure of compensation.

Similar to the results obtained for *Atox1*^{−/−} mouse fibroblasts, where loss of *Atox1* leads to a reduced cellular efflux of copper on the one hand and a reduced influx of cisplatin on the other hand (Safaei et al. 2009), we observe a reduced sensitivity of the *Atox1* mutant flies to cisplatin. Why *Atox1* mutants are less affected by cisplatin relative to controls is still unclear. It was suggested that cisplatin is taken up via internalization and degradation of Ctr1 in a process that also depends on Atox1 (Safaei et al. 2009). Since Atox1 by itself binds cisplatin (Boal and Rosenzweig 2009) its cellular distribution might be hampered in *Atox1*^{−/−} cells. Additionally, as copper accumulates in the intestinal cells of *Atox1* mutant flies, Ctr1B protein levels might be constantly low in these cells and thus not allow for efficient cisplatin uptake. Unlike in mammalian cells (Safaei et al. 2009) and *Drosophila*, as shown here, the sensitivity

to cisplatin is not altered in a yeast mutant lacking *ATX1* (Ishida et al. 2002).

The copper transporters Ctr1, Ctr2, ATP7A and ATP7B were shown to regulate the cellular pharmacology of cisplatin and their altered expression or localization is involved in tumor resistance (Gupta and Lutsenko 2009). The data presented here, together with the results obtained by Safaei and coworkers (2009), suggest that also downregulation of Atox1 may be a mechanism that contributes to cisplatin resistance in some cancer cells.

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